

## REMARKS

The applicant respectfully requests that the mailing date of the Office Action, paper number 18, be changed from September 9, 2003, to October 6, 2003, which is the date it was faxed by the Patent Office to the applicant's attorney of record. The initial document was not sent by the Patent Office to the attorney of record, but instead to Genencor International.

Currently pending in the application are claims 1-5, 7-8, 11-16, 19-22, 24-25, and 27-30 and 32-35. Claims 17 and 26 were cancelled in a prior amendment. Claims 6, 9, 10, 17, 18, 23 and 31 are cancelled in this amendment.

The Applicant has adopted the Examiner's suggestions in claims 8 and 21 to use "and wherein" for clarification and has inserted the wash conditions for highly stringent hybridization conditions into claims 11 and 24. The unnecessary phrase "commercial levels" is deleted from claims 15 and 25, and claim 16 amendment to property depend from claim 15. Claim 25 is amended to recite production of a biomass and to recite production of laccase, also for clarification and consistency.

The claims have been rejected, the Examiner noting that while the specification teaches production of laccase in a plant by seed-preferred expression, the claim language to that affect should be included in the claims. Claims 1, 15 and 25, and accordingly all of the claims depending from same (2-5, 7, 8, 11-14, 16, 19-22, 24, 27-30, 32-35) include that language. By preferentially directing expression of laccase to the seed of the plant the plant is protected from adverse affects of the laccase, and thus can accumulate laccase without dying. Further, when the laccase is also secreted to the cell wall, expression can be enhanced as well. Thus, new claim 34 depends from claim 1, and in addition to reciting expression to the seed of the plant, also recites the laccase expression is directed to the cell wall. New claim 35 depends from claim 25, and likewise recites a method where the laccase is secreted to the cell wall. Claim 16 recites the method of producing laccase in plants where the laccase is secreted to the cell wall. Thus it is believed these claims are in condition for allowance.

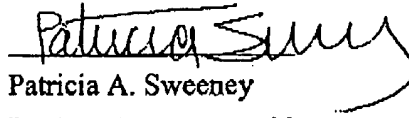
New claims 32 and 33 dependent from claims 1 and 15 respectively, and recite laccase production levels of about 0.01%. This language was simply moved to a dependent claim.

The Examiner has noted that it appears only plant and fungal laccase have been characterized. The applicant points out that the source of the laccase-encoding gene is not critical to the invention. The invention addresses the problem of how a plant can express laccase, regardless of what gene may be making the laccase, without the plant dying. However, there are laccase-encoding genes which have been characterized from other sources. Attached to this amendment are journal articles showing laccase-encoding genes from bacteria (Aria et al. "Kraft pulp biobleaching and mediated oxidation of a nonphenolic substrate by laccase from *Streptomyces cyaneus* CECT335" *Applied and Environ. Microbiology* Apr. 2003 pp. 1953-1958; Enguita et al, "Crystal structure of a bacterial endospore coat component, a laccase with enhanced thermostability properties" *J. Biol. Chem.* 278:19416-19425, May 23, 2003 Genbank 1GSK\_A; Genbank AB092576, *Streptomyces lavendulae* gene for laccase); and from worms (Pubmed accession No. NM\_069101, *Caenorhabditis elegans* laccase (4J499), see [www.ncbi.nlm.nih.gov:80/entrez/viewer](http://www.ncbi.nlm.nih.gov:80/entrez/viewer)). Thus, a wide range of laccase sources are available to choose from: fungus, plant, bacteria and worms. The particular source is irrelevant; what is important is that the sequence encodes laccase. The Examiner also queries about a reference to support the conclusion that prior attempts to express higher levels of laccase in plant have not been successful. While it is difficult to find a reference showing lack of an activity, the applicant points out page three of the declaration of Dr. Hood, submitted in the prior amendment, in which Dr. Hood reports that prior attempts to express laccase resulted in very low expression or death of the plants, and further refers to page 138 of Hood et al. *Plant Biotechnology Journal* (2003) 1, pp129-140.

The amendments submitted do not introduce new matter and are responsive to new comments and/or requests of the Examiner. It is submitted the amendment places the claims in condition for allowance or better condition for appeal. For these reasons, reconsideration and allowance of the claims is respectfully requested. The Examiner is

invited to contact the attorney of record below with any questions or concerns.

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## Kraft Pulp Biobleaching and Mediated Oxidation of a Nonphenolic Substrate by Laccase from *Streptomyces cyaneus* CECT 3335

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A new laccase (EC 1.10.3.2) produced by *Streptomyces cyaneus* CECT 3335 in liquid media containing soya flour (20 g per liter) was purified to homogeneity. The physicochemical, catalytic, and spectral characteristics of this enzyme, as well as its suitability for biobleaching of eucalyptus kraft pulps, were assessed. The purified laccase had a molecular mass of 75 kDa and an isoelectric point of 5.6, and its optimal pH and temperature were 4.5 and 70°C, respectively. The activity was strongly enhanced in the presence of Cu<sup>2+</sup>, Mn<sup>2+</sup>, and Mg<sup>2+</sup> and was completely inhibited by EDTA and sodium azide. The purified laccase exhibited high levels of activity against 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) and 2,6-dimethoxyphenol and no activity against tyrosine. The UV-visible spectrum of the purified laccase was the typical spectrum of the blue laccases, with an absorption peak at 600 nm and a shoulder around 330 to 340 nm. The ability of the purified laccase to oxidize a nonphenolic compound, such as veratryl alcohol, in the presence of ABTS opens up new possibilities for the use of bacterial laccases in the pulp and paper industry. We demonstrated that application of the laccase from *S. cyaneus* in the presence of ABTS to biobleaching of eucalyptus kraft pulps resulted in a significant decrease in the kappa number (2.3 U) and an important increase in the brightness (2.2%, as determined by the International Standard Organization test) of pulps, showing the suitability of laccases produced by streptomycetes for industrial purposes.

Because of their potential for biotechnological applications in areas such as biobleaching, increasing the strength of cellulose fibers, textile dye or stain bleaching, and bioremediation, attention is currently being paid to laccases (2, 23, 30, 43, 50). These enzymes are widely distributed in plants and fungi, but until now laccase activity has been reported in only a few bacteria, including *Azospirillum lipoferum*, *Marinomonas mediterranea*, *Streptomyces griseus*, and *Bacillus subtilis* (1, 15, 20, 22, 41, 45). A number of roles for laccases in bacterial systems have been suggested and include roles in melanin production and spore coat resistance and involvement in morphogenesis (15, 22). In *Streptomyces cyaneus*, a laccase-type phenol oxidase was found to be produced during growth under solid-substrate fermentation conditions, and it was suggested that this enzyme was involved in the solubilization and mineralization of lignin from wheat straw (5). Further studies demonstrated that this organism could be used to improve the qualities of pulp after 2 weeks of incubation under solid-substrate fermentation conditions (6). However, to date there have been no reports describing the involvement of bacterial laccases in the oxidation of nonphenolic compounds in the presence of mediators. The specific activities of these enzymes with lignin and related substrates have not been examined yet.

Laccases are considered some of the most promising enzymes for future industrial applications in the pulp and paper industry (4, 10, 31, 33, 38). In particular, laccases from fungi have been found to reduce the kappa number and enhance the

bleaching of kraft pulp when they are used in the presence of chemical mediators, such as 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) (8, 11, 49). In this paper we describe the behavior of the phenol oxidase produced by *S. cyaneus* CECT 3335 in submerged cultures as a laccase-type behavior, and in this study we also confirmed the usefulness of the purified enzyme in the biobleaching of kraft pulps.

### MATERIALS AND METHODS

**Microorganism and growth conditions.** *S. cyaneus* CECT 3335 was maintained as a suspension of spores and hyphal fragments in 20% glycerol at -70°C and was routinely cultured on Bennet agar (29) or soya flour mannitol agar containing (per liter) 20 g of soya flour, 20 g of mannitol, and 20 g of agar. Distilled water suspensions of sporulating growth (10<sup>7</sup> CFU ml<sup>-1</sup>) were used as inocula. Standard spore suspensions (2 ml) were used to inoculate 500-ml flasks containing 100 ml of basal mineral medium (13) supplemented with soya flour (20 g per liter). Cultures were shaken at 200 rpm and incubated at 28°C for 10 days. Flasks were removed every 24 h to determine the enzyme activity. Culture supernatants were obtained by centrifugation of the contents of flasks at 10,000 × g at 4°C for 10 min. Cell-free culture supernatants were stored at -20°C until they were used.

**Enzyme assays and protein determination.** Laccase activity was determined by determining the oxidation of 5 mM ABTS (Sigma-Aldrich) (48) in 0.1 M Mcllvaine buffer (pH 4.4). The increase in absorbance at 436 nm was monitored with a Bockman DU-50 spectrophotometer connected to an Ultratherm thermostatic bath, which maintained the temperature of the reaction mixture at 60 ± 2°C. To calculate enzymic activity, we used an absorption coefficient of 29,500 M<sup>-1</sup> cm<sup>-1</sup> for oxidized ABTS.

The time course of growth was estimated by measuring intracellular protein concentrations. Cells were obtained daily by centrifugation at 5,000 × g for 10 min, and then they were washed with distilled water and resuspended in 10 ml of 0.1 M sodium phosphate buffer (pH 6.8). The cells were disrupted with a French pressure cell (SLM AMINCO), and the intracellular protein content was estimated by the Bradford method (9).

Protein contents in the supernatant were estimated as described above, and the specific activities were expressed as milliunits per minute per milligram of culture supernatant protein.

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**Purification and characterization of laccase.** Proteins were precipitated from the supernatant by addition of ammonium sulfate (up to 50% saturation) at 4°C and centrifugation at  $10,000 \times g$  for 30 min. The precipitate was resuspended in 0.1 M sodium phosphate buffer (pH 6.8) and extensively dialyzed at 4°C against the same buffer. The sample was precipitated again with ammonium sulfate (up to 20%) and centrifuged as described above.

Liquid chromatography was carried out with a GP-250 Plus fast-performance liquid chromatographic system (Pharmacia). Supernatant (2 ml) was loaded onto a hydrophobic column (Econo-Pac Methyl cartridge; Bio-Rad). The column was equilibrated with 0.1 M phosphate buffer (pH 6.8) supplemented with 1.1 M ammonium sulfate. The adsorbed proteins were eluted with the same buffer without ammonium sulfate by using a continuous gradient (1.1 to 0 M ammonium sulfate) at a flow rate of  $1 \text{ ml min}^{-1}$ . Fractions with laccase activity were pooled, concentrated, and applied to an anion-exchange column (Econo-Pac Q cartridge; Bio-Rad). In this step the column was equilibrated with 0.1 M phosphate buffer (pH 6.8), and the adsorbed proteins were eluted with the same buffer by using the following NaCl gradient: 35% NaCl (7 min) and 100% NaCl (5 min) at a flow rate of  $1 \text{ ml min}^{-1}$ . Chromatograms were obtained by monitoring the absorbance at 280 nm. The ABTS-laccase activity and proteins were assayed in all fractions collected (1 ml).

Throughout the purification process, fractions were analyzed by native and denaturing polyacrylamide gel electrophoresis (PAGE).

Sodium dodecyl sulfate (SDS)-PAGE on 9% polyacrylamide gels was performed as described by Laemmli (27). Culture supernatants were obtained daily, and native PAGE (9% polyacrylamide) was used to separate the extracellular proteins. Gels were developed as zymograms for laccase activity by using ABTS as the substrate. After electrophoresis, the gels were overlaid onto a 1.5% (wt/vol) agarose gel sheet (thickness, 1 mm) containing 5 mM ABTS in 0.1 M Mcllvaine buffer (pH 4.5). Both the polyacrylamide gel and the agarose gel were placed on a glass plate and incubated for 15 to 30 min at 45°C. During the purification process, SDS-PAGE analysis was also carried out. Protein bands were stained with Coomassie brilliant blue R-250 or silver stain (Bio-Rad) and compared with the bands of molecular weight markers (Bio-Rad). Isoelectric focusing was carried out by using a Bio-Rad mini isoelectric focusing cell system according to the manufacturer's instructions at pH 3 to 9; standard Bio-Rad markers were included. Protein bands were visualized by Coomassie brilliant blue R-250 staining.

Estimates of the optimal temperature and pH for activity and estimates of the thermal and pH stabilities of the purified laccase were obtained by using a temperature range of 30 to 100°C and a pH range of 3 to 8 with 10 mM Mcllvaine buffer (pH 3 to 7) and 10 mM phosphate buffer (pH 7 to 8).

Michaelis-Menten kinetic parameters ( $K_m$  and  $V_{max}$ ) for the purified laccase ( $481 \text{ } \mu\text{g ml}^{-1}$ ) were determined at the optimal temperature (70°C) in 0.1 M Mcllvaine buffer (pH 4.5) by using ABTS at concentrations of 0 to 50 mM as the substrate.

The effects of metal ions ( $\text{CuSO}_4$ ,  $\text{MnSO}_4$ ,  $\text{MgSO}_4$ ,  $\text{ZnCl}_2$ ,  $\text{CaCl}_2$ , and  $\text{FeSO}_4$ ) at concentrations ranging from 0 to 50 mM were also determined. In addition, the effects of EDTA (0 to 50 mM), sodium azide (0 to 100 mM), and other potential inhibitors (phenylthiourea, cinnamic acid, and tropolone, each at a concentration of 10 mM; Sigma-Aldrich) were also examined. Additional assays with metal ions ( $\text{CuSO}_4$ ,  $\text{MnSO}_4$ , and  $\text{MgSO}_4$ ) at concentrations of 0 to 50 mM were performed in order to determine their effects on the recovery of the activity inhibited by EDTA.

Spectrophotometric measurements of substrate oxidation by the purified enzyme were obtained at the optimal pH and optimal temperature by using 1-ml reaction mixtures containing the test substrates L-tyrosine (475 nm), L-3,4-dihydroxyphenylalanine (475 nm), tyramine (460 nm), dopamine (475 nm), protocatechuic acid (400 nm), ABTS (436 nm), 2,6-dimethoxyphenol (2,6-DMP) (468 nm), guaiacol (470 nm), and syringaldazine (525 nm) dissolved in 0.1 M Mcllvaine buffer. Most of the substrates were used at a concentration of 5 mM; the only exception was syringaldazine, which was used at a concentration of 0.1 mM. For substrate specificity, enzymatic activity was expressed in absorbance units (AU), and 1 AU was defined as the amount of enzyme required to increase the absorbance at each characteristic wavelength indicated above by 1 U per min per ml. All chemicals were purchased from Sigma-Aldrich.

The UV-visible absorption spectrum of the purified laccase produced by *S. cyaneus* was determined at wavelengths between 700 and 200 nm at room temperature in 50 mM phosphate buffer (pH 6) by using a Beckman DU 50 spectrophotometer.

Oxidation of veratryl alcohol by the purified laccase with ABTS as the mediator. Oxidation of veratryl alcohol (3,5-dimethoxybenzyl alcohol) by the purified enzyme in the presence of ABTS was carried out by using the following stock solutions: purified laccase ( $2.5 \text{ U ml}^{-1}$ ), veratryl alcohol (5 mM), ABTS (5 mM),

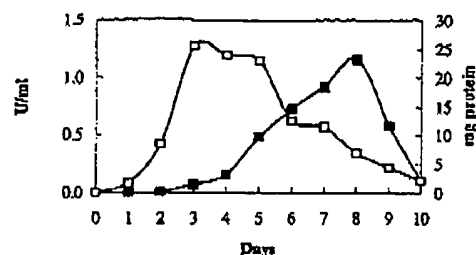


FIG. 1. Growth of *S. cyaneus* (as determined by intracellular protein content) (□) and production of extracellular laccase activity (■) over 10 days in basal mineral medium supplemented with soya flour (2 g per liter).

and Mcllvaine buffer (0.1 M, pH 4.5). The standards used (each at a concentration of 1 mM) were veratryl alcohol, veratraldehyde (3,5-dimethoxybenzaldehyde), and veratric acid (3,5-dimethoxybenzoic acid). The controls contained laccase (10  $\mu\text{l}$ ), veratryl alcohol (50  $\mu\text{l}$ ), and buffer (40  $\mu\text{l}$ ). The samples contained laccase (10  $\mu\text{l}$ ), veratryl alcohol (50  $\mu\text{l}$ ), ABTS (30  $\mu\text{l}$ ), and buffer (10  $\mu\text{l}$ ). The samples were incubated at room temperature for 24, 36, and 48 h and 5 days in the dark. At the end of the incubation period, samples were centrifuged at  $10,000 \times g$  for 5 min. Each sample (10  $\mu\text{l}$ ) was analyzed by high-performance liquid chromatography (LC-9A; Shimadzu) by using a 25-cm column (Nucosil 10-5 C<sub>18</sub>). The column was previously equilibrated with 15% (vol/vol) acetonitrile and a solution containing phosphoric acid (0.02 M) and NaOH (0.02 M) at pH 3 and was eluted at a rate of  $1 \text{ ml min}^{-1}$  with the following acetonitrile gradient: 15% acetonitrile (1 min), 35% acetonitrile (10 min), 70% acetonitrile (15 min), and 97% acetonitrile (16 min; maintained until 18 min). Eluted compounds were monitored at 310 nm by photodiode detection (Waters 996). Standard solutions containing different concentrations (0.1 to 10 mM) of veratryl alcohol, veratraldehyde, and veratric acid were chromatographed under the conditions described above, and calibration curves were obtained. Quantification of the compounds was carried out by integrating the area under each peak at 280 nm. All chemicals were obtained from Sigma-Aldrich.

**Application of purified laccase to the biobleaching of eucalyptus kraft pulp.** Purified laccase produced by *S. cyaneus* was evaluated for biobleaching of eucalyptus kraft pulps by using ABTS as the mediator. The eucalyptus kraft pulp used in this assay was soaked with distilled water for 4 h at room temperature and then filtered and dried at 90°C to obtain a constant dry mass.

Enzymatic bleaching of the pulp with laccase and laccase-mediator systems was carried out in 500-ml Erlenmeyer flasks containing 7.5 g of pulp, 100 mM (16  $\mu\text{g}$ ) of purified laccase per g of pulp, and (in the laccase-mediator system assay) 5 mM ABTS. The volume of each assay mixture was adjusted to 100 ml with 50 mM Mcllvaine buffer (pH 5) to obtain a final pulp content of 7.5% (wt/vol). The reaction mixtures were incubated at 45°C for 3 h with shaking and were periodically oxygenated. Control assay mixtures were prepared by using heat-inactivated enzyme.

Controls and pulps treated with enzyme and with the enzyme-mediator system were further extracted with 37.5 mM NaOH at 80°C for 3 h, and the kappa number (ISO-302-1981 E) was determined. After alkaline extraction, controls and treated pulps were further bleached by peroxide extraction. Peroxide bleaching of pulps was carried out in 250-ml Erlenmeyer flasks containing 2.5% (vol/vol)  $\text{H}_2\text{O}_2$  (obtained from a 33%  $\text{H}_2\text{O}_2$  [wt/vol] commercial solution), 0.05% (wt/vol)  $\text{MgSO}_4$ , and 3.5% (wt/vol)  $\text{Na}_2\text{SiO}_3$  in an appropriate volume of distilled water to obtain a final pulp content of 10% (wt/vol). The peroxide extraction was carried out at 60°C for 1 h with shaking. Finally, the kappa number and brightness (ISO 2496) were determined. All the assays were performed in triplicate.

## RESULTS

**Production of laccases in submerged culture.** The time course for the production of extracellular laccase activity during the growth of *S. cyaneus* in submerged culture is shown in Fig. 1. The maximum laccase activity ( $1.2 \text{ U ml}^{-1}$ ) was obtained after 8 days of growth. The maximum growth, as determined by using intracellular protein, was detected after 3 days.

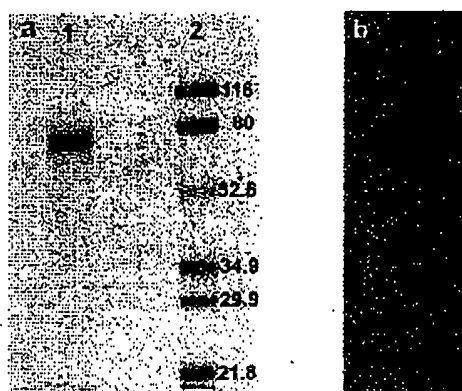


FIG. 2. (a) SDS-PAGE of purified laccase produced by *S. cyaneus* stained with Coomassie brilliant blue (lane 1) and molecular mass markers (lane 2). (b) Zymogram of laccase stained with ABTS. Electrophoresis was performed after 25  $\mu$ g of protein was loaded.

After 8 days the intracellular protein concentration had declined to 6.8 mg ml<sup>-1</sup> (Fig. 1).

**Purification of laccase.** The laccase activity in the culture supernatant (100 ml) obtained after 8 days of growth (approximately 14 mg) was purified by hydrophobic interaction chromatography followed by anion-exchange chromatography as described in Materials and Methods. At the end of the purification process a purified laccase (Fig. 2) was obtained; this laccase had a specific activity of 6.3 U mg of protein<sup>-1</sup>, and the purification factor was 6.16-fold, which corresponded to a final yield of 57.8% (Table 1).

**Characterization of the purified laccase.** The purified laccase produced one band on an SDS-PAGE gel at a molecular mass of approximately 75 kDa (Fig. 2). The isoelectric point for this laccase was 5.6. The optimum pH for the enzyme was 4.5, and the optimum temperature for the purified laccase was 70°C. The enzyme retained up to 100% of its activity after 120 min of incubation at 40°C at pH values between 5 and 8 and lost 50% of its activity when it was incubated at pH 3 to 4. The laccase retained more than 75% of its activity after incubation for 120 min at 50°C and 60% of its activity after incubation at 60°C for 60 min at pH 4.5. The laccase activity was enhanced in the presence of CuSO<sub>4</sub> at concentrations up to 10 mM (maximum activity at 10 mM), in the presence of MnSO<sub>4</sub> at concentrations up to 40 mM (maximum activity at 20 mM), and in the presence of MgSO<sub>4</sub> at concentrations up to 40 mM (maximum activity at 10 mM) and was reduced in the presence of FeSO<sub>4</sub>, ZnCl<sub>2</sub>, and CaCl<sub>2</sub> at the range of concentrations assessed (up to 50 mM).

Inhibition of laccase activity was observed in the presence of 10 mM tropolone (31.0% inhibition), phenylthiourea (95.0%), and cinnamic acid ( $\alpha$ -phenylpropenoic acid) (75.7%). In the presence of EDTA and sodium azide, complete inhibition was detected at concentrations of 30 and 20 mM, respectively. In the case of EDTA inhibition, activity was recovered after addition of CuSO<sub>4</sub> (100% recovery at a concentration of 40 mM), MnSO<sub>4</sub> (150% recovery at a concentration of 35 mM), or MgSO<sub>4</sub> (30% recovery at a concentration of 40 mM).

The kinetic constants of the purified laccase were determined under the optimal assay conditions. The  $K_m$  was 0.38 mM, the  $V_{max}$  for ABTS was 5.55 U mg of protein<sup>-1</sup>, and the efficiency of the enzyme against ABTS was 14.60 U mg of protein<sup>-1</sup> mg of substrate<sup>-1</sup>.

In terms of substrate specificity, the purified laccase showed high activity against ABTS (0.763 AU 100  $\mu$ g of protein<sup>-1</sup>) and 2,6-DMP (0.830 AU 100  $\mu$ g of protein<sup>-1</sup>), lower activity against syringaldazine (0.03 AU 100  $\mu$ g of protein<sup>-1</sup>) and protocatechuic acid (0.028 AU 100  $\mu$ g of protein<sup>-1</sup>), and very low activity against tyramine (0.01 AU 100  $\mu$ g of protein<sup>-1</sup>), L-3,4-dihydroxyphenylalanine (0.015 AU 100  $\mu$ g of protein<sup>-1</sup>), dopamine (0.01 AU 100  $\mu$ g of protein<sup>-1</sup>), and guaiacol (0.005 AU 100  $\mu$ g of protein<sup>-1</sup>). No activity against tyrosine was detected.

The UV-visible spectrum of the purified laccase had a peak of absorption at around 600 nm and a shoulder at around 330 to 340 nm (Fig. 3).

**Effect of the purified enzyme on the oxidation of veratryl alcohol when ABTS was used as the mediator.** The results obtained after 5 days of incubation are shown in Fig. 4. A decrease in the relative area of veratryl alcohol was observed along with an increase in the concentration of veratraldehyde. In addition, a small increase in the veratric acid concentration was detected over the first 36 h. In all control experiments veratraldehyde and veratric acid were not detected.

**Application of purified laccase to biobleaching of eucalyptus kraft pulps.** The effect of the purified laccase on biobleaching of eucalyptus kraft pulp was evaluated after the most common pulp properties, kappa number and brightness, were analyzed (Fig. 5). Following pulp treatment with laccase supplemented with ABTS and further alkaline extraction, a reduction in the kappa number of 2.3 U was observed (Fig. 5a). When laccase was used by itself (i.e., in the absence of the mediator ABTS), a reduction in the kappa number of only 0.4 U (compared with the control) was observed. In addition, laccase treatment followed by alkaline extraction yielded pulp with substantially increased brightness (1.1-U increase). Application of laccase plus ABTS as the mediator resulted in even greater increases in brightness (2.2 U compared with the control) after alkaline

TABLE 1. Purification of laccase produced by *S. cyaneus*

Purification step	Total protein (mg)	Total activity (U)	Sp act (U mg of protein <sup>-1</sup> )	Purification (fold)	Yield (%)
Initial supernatant, (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	87.67	88.33	1.02	1	100
Precipitation (50%), (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	62.76	83.71	1.33	1.3	93.7
Precipitation (20%)	45.8	70.84	1.55	1.52	79.3
Econo Methyl	21.44	52.53	2.44	2.39	58.8
Econo Q	8.19	51.64	6.3	6.16	57.8

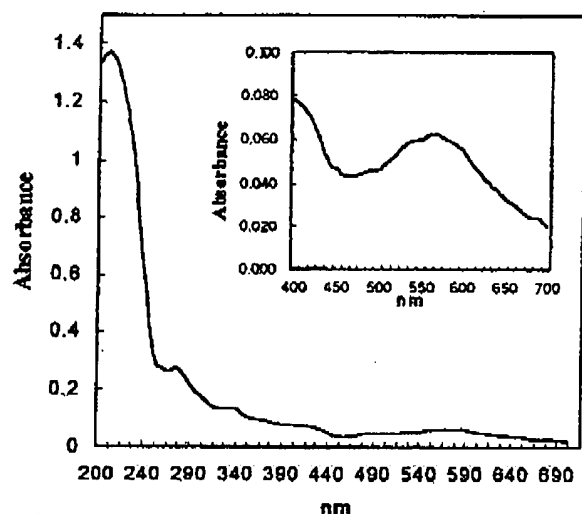


FIG. 3. UV-visible spectrum of laccase produced by *S. cyaneus*. The spectrum was obtained by using 25  $\mu$ g of purified laccase per ml.

extraction (Fig. 5b). After  $H_2O_2$  was applied to control pulp, a significant reduction in the kappa number (3.8 U) was observed. Following pulp treatment with both laccase and laccase plus ABTS in a bleaching sequence with  $H_2O_2$ , considerable reductions in the kappa number were observed (0.3 and 1.4 U, respectively). Increases in brightness were also observed after  $H_2O_2$  was applied to the pulps treated with enzyme and with enzyme plus ABTS (1.1 and 2.2 U compared with the control) (Fig. 5b).

## DISCUSSION

The present work was initiated by studying the production kinetics of laccase activity from *S. cyaneus* in submerged cultures by using soya medium. Although maximum growth was observed after 3 days of incubation, extracellular laccase activity was detected after 4 days of growth, and the maximum extracellular laccase activity was detected after 8 days of incubation, when the specific activity was 250 mU mg of protein<sup>-1</sup>, a value which is slightly greater than previously reported bacterial laccase activities (40, 41). This pattern shows that laccase activity can be detected once a strain has reached the stationary phase of growth and increases with cell autolysis. In general, the bacterial laccases that have been described are intracellular or cell bound. Furthermore, most of them are involved in basic cellular processes (e.g., the *Azospirillum* and *Marinomonas* enzymes and CotA from *B. subtilis*) (18, 19, 30). Recently, an extracytoplasmic phenol oxidase produced by the actinomycete *S. griseus* was described, and it has been suggested that this enzyme may be involved in the onset of morphogenesis (15). Therefore, to date, there have been no previous studies based on purified enzymes to determine the potential applications of bacterial laccases in the pulp industry, and in this paper we describe for the first time the use of a streptomycete laccase in biobleaching.

The purification procedure used for the laccase in this study

resulted in sixfold purification and a yield of 58%. The molecular mass (75 kDa) and the isoelectric point (pI 5.6) reported here are different from those reported for the intracellular laccases of *B. subtilis* (molecular mass, 65 kDa; pI 7.7) (30) and *S. griseus* (molecular mass, 100 kDa) (15). However, the values which we obtained are similar to the previously described values for extracellular lignin-degrading laccases from the fungi *Pleurotus ostreatus* (35, 36) and *Marasmius quercophilus* (16).

The results which we obtained for the stability of the laccase at a high temperature and the optimum temperature can be considered industrially advantageous when they are compared with the results obtained for laccases produced by other organisms. The optimal temperature range for fungal laccases is 30 to 60°C (14, 32, 42). The enzyme activity of our purified laccase was maintained in the pH range determined for other fungal and bacterial laccases. In comparison, the optimum pH for the laccase activity from *B. subtilis* was reported to be pH 3 when ABTS was used as the substrate (30). However, no other comparative data were found for the stability of streptomycete laccases at different pH values and temperatures.

The effects of different metal ions on the laccase activity showed that copper, manganese, and magnesium activated the purified laccase, while ferric iron, calcium, and zinc reduced the activity. No equivalent data for bacterial laccase activity has been published. However, a copper requirement for bacterial laccase activity has been reported previously for *S. griseus* (15), *B. subtilis*, and *A. lipoferrum* (20).

The effects of inhibitors showed that the purified laccase was sensitive to the inhibitors tropolone, phenylthiourea, and cinnamic acid. The observed responses to these inhibitors appear to be a unique feature of the purified laccase; this enzyme is similar to tyrosinases in terms of its sensitivity to tropolone and cinnamic acid (25, 47) and differs from fungal laccases (18). Nevertheless, the possibility that the behavior of phenol oxidases with different inhibitors could be affected by the conditions of the assay must be considered. The observation that EDTA completely inhibited laccase activity, which could then be recovered after addition of either copper, manganese, or magnesium, again suggests that bacterial laccases have a unique feature (12). Moreover, the enzyme was also inhibited by sodium azide, as described previously for other bacterial and fungal laccases (18, 20, 28).

Regarding substrate specificity, the laccase from *S. cyaneus*

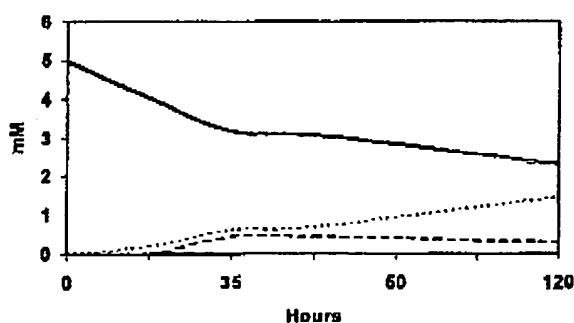


FIG. 4. Oxidation of veratryl alcohol by laccase produced by *S. cyaneus* in the presence of ABTS. Solid line, veratryl alcohol; dashed line, veratraldehyde; dotted line, veratric acid.

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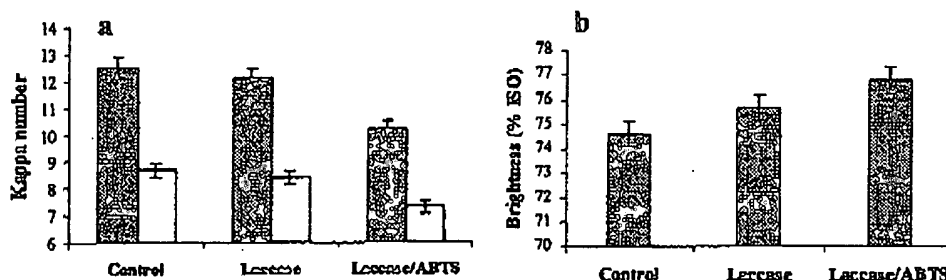


FIG. 5. (a) Kappa number values of eucalyptus kraft pulp after enzymatic treatment with laccase produced by *S. cyaneus* followed by alkaline extraction (grey bars) and alkaline and peroxide extraction (open bars). (b) Brightness (expressed as a percentage, as determined by the International Standard Organization [ISO] test) of eucalyptus kraft pulp after enzymatic treatment with laccase produced by *S. cyaneus* followed by alkaline and peroxide extraction.

showed strong activity against two substrates which are considered highly specific for laccases (ABTS and 2,6-DMP) and showed much lower activity at the pH used against syringaldazine and guaiacol, which are also considered specific substrates for these enzymes (19). Nevertheless, difficulty oxidizing guaiacol has been also reported for some specific laccases (40, 44). The enzyme also showed low specificity against *o*-diphenolic substrates, such as protocatechuic acid, which is considered a substrate for catecholase (19). No activity against tyrosine was detectable; this is interesting because tyrosinases are the most common phenol oxidases produced by streptomycetes (26).

The UV-visible spectrum of the purified laccase was the typical spectrum of the blue laccases (with a peak at around 600 nm and a shoulder at around 330 nm). In fact, typical laccases isolated from fungi belong to the class containing the blue oxidases, which contain four copper atoms per molecule arranged in three different copper-binding sites (39, 46). Nevertheless, recent work on the laccase from *B. subtilis* determined that this oxidase has four copper-binding sites (22). All of these sites produce spectra with a maximum at 605 nm, which corresponds to a type 1 or blue copper atom. Type 2 copper exhibits weakly visible absorbance, and type 3 copper has two copper centers and is responsible for a shoulder at around 330 nm in the absorbance spectrum of native laccase (35, 37). A bacterial laccase, the Cota protein from *B. subtilis*, also showed the typical UV-visible spectrum of blue multicopper oxidases (30). The cloned gene corresponding to the multipotent phenol oxidase of *M. mediterraneus* also had the motifs corresponding to the four copper-binding sites characteristic of most laccases (41).

The oxidation of veratryl alcohol by the purified laccase in the presence of ABTS described here is the first oxidation of a nonphenolic substrate by a purified bacterial laccase that has been reported. This activity opens up the potential for using this enzyme-mediator system in the pulp and paper industry as an alternative to fungus-based systems (7). A number of researchers have focused their efforts on description and characterization of laccases produced by lignin-degrading fungi. Although these enzymes have limited abilities to degrade lignin, in the presence of oxygen and an organic compound as an electron carrier (or mediator), they are able to delignify kraft pulps. This delignification of kraft pulps has been patented and

named the Laccase Mediator System (LMS) (10). This LMS is highly selective and yields pulp with little carbohydrate loss or damage (49). The success achieved at the laboratory and pilot-plant scales (11, 34) makes this process a desirable system for industrial applications of bleaching of kraft pulps for the manufacture of paper (17).

In this work we demonstrated that laccase produced by *S. cyaneus* can delignify eucalyptus kraft pulp in the presence of ABTS as a mediator. The results which we obtained are comparable to those described by other workers who used different laccases produced by ligninolytic fungi and ABTS as the mediator. The level of delignification achieved with the LMS of *S. cyaneus* was an 18.4% reduction in the kappa number after alkaline extraction of the pulp. Taking into consideration the fact that the biobleaching experiment was carried out with 100 mU per g of pulp during a 2-h treatment, this result can be compared to the decrease in the kappa number of a softwood kraft pulp treated with 5 U of laccase produced by *Trametes versicolor* per g of pulp (3) and also with the decrease obtained by using 100 mU of the same laccase after 1 day of incubation (7). Moreover, the reduction in the kappa number obtained with the LMS of *S. cyaneus* after peroxide extraction was similar to the reduction obtained with a commercial laccase from Novo Nordisk (49). Moreover, the increase in brightness obtained after addition of hydrogen peroxide to LMS-treated pulps was significant. This result also confirms the potential application of the laccases from streptomycetes to biobleaching of kraft pulps in the presence of a synthetic mediator. Because the use of ABTS as a mediator for biobleaching of pulps is not considered cost-effective at an industrial scale, the search for natural mediators is a current research priority. The detection of low-molecular-weight aromatic compounds, such as *p*-hydroxybenzoic acid, after growth of streptomycetes on wheat straw (21) could open new possibilities for the use of natural mediators together with bacterial laccases for biobleaching. In fact, *p*-hydroxybenzoic acid has been considered a mediator as efficient as ABTS for the oxidation of polycyclic aromatic hydrocarbons (24).

In conclusion, we isolated, purified, and characterized a new extracellular bacterial laccase. Evidence for this was obtained from substrate specificity data and the kinetic, biochemical, and spectral characteristics combined with the ability of the enzyme to oxidize nonphenolic substrates in the presence of



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ABTS as a mediator. For the first time, the potential of laccases from streptomycetes for use in combination with mediators for biobleaching of kraft pulps has been demonstrated.

## ACKNOWLEDGMENTS

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### Crystal Structure of a Bacterial Endospore Coat Component

#### A LACCASE WITH ENHANCED THERMOSTABILITY PROPERTIES\*

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Endospores produced by the Gram-positive soil bacterium *Bacillus subtilis* are shielded by a proteinaceous coat formed by over 30 structural components, which self-assemble into a lamellar inner coat and a thicker striated electrodense outer coat. The 65-kDa CotA protein is an abundant component of the outer coat layer. CotA is a highly thermostable laccase, assembly of which into the coat is required for spore resistance against hydrogen peroxide and UV light. Here, we report the structure of CotA at 1.7-Å resolution, as determined by x-ray crystallography. This is the first structure of an endospore coat component, and also the first structure of a bacterial laccase. The overall fold of CotA comprises three cupredoxin-like domains and includes one mononuclear and one trinuclear copper center. This arrangement is similar to that of other multicopper oxidases and most similar to that of the copper tolerance protein CueO of *Escherichia coli*. However, the three cupredoxin domains in CotA are further linked by external interdomain loops, which increase the packing level of the structure. We propose that these interdomain loops contribute to the remarkable thermostability of the enzyme, but our results suggest that additional factors are likely to play a role. Comparisons with the structure of other monomeric multicopper oxidases containing four copper atoms suggest that CotA may accept the largest substrates of any known laccase. Moreover, and unlike other laccases, CotA appears to have a flexible lidlike region close to the substrate-binding site that may mediate substrate accessibility. The implications of these findings for the properties of CotA, its assembly and the properties of the bacterial spore coat structure are discussed.

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PubMed Nucleotide Protein Genome Structure PM

1GSKA. Chain A, Crystal ...[gi:31615302] BLink, Domains, Links

LOCUS 1GSK\_A 513 aa linear BCT 08-JAN-2002

DEFINITION Chain A, Crystal Structure Of Cota, An Endospore Coat Protein From  
Bacillus Subtilis.

ACCESSION 1GSK\_A

VERSION 1GSK\_A GI:31615302

DBSOURCE pdb: molecule 1GSK, chain 65, release Jan 8, 2002;

deposition: Jan 8, 2002;

class: Sporulation;

source: Mol\_id: 1; Organism scientific: Bacillus Subtilis;

Expression\_system: Escherichia Coli; Expression\_system\_strain:  
B121;

Exp. method: X-Ray Diffraction.

KEYWORDS

SOURCE Bacillus subtilis

ORGANISM Bacillus subtilis

Bacteria; Firmicutes; Bacillales; Bacillaceae; Bacillus.

REFERENCE 1 (residues 1 to 513)

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TITLE Direct Submission

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COMMENT Revision History:

MAY 21 3 Initial Entry.

FEATURES Location/Qualifiers

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2: AB092576: Streptomyces lave...[gi:23491745] Links

LOCUS AB092576 2318 bp DNA linear BCT 28-SEP-2002

DEFINITION Streptomyces lavendulae gene for laccase, complete cds.

ACCESSION AB092576

VERSION AB092576.1 GI:23491745

KEYWORDS

SOURCE Streptomyces lavendulae

ORGANISM Streptomyces lavendulae

Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales;

Streptomycineae; Streptomycetaceae; Streptomyces.

REFERENCE 1

AUTHORS Suzuki,T., Ito,M., Tsujibo,H., Miyamoto,K. and Inamori,Y.

TITLE A Thermostable Laccase from Streptomyces lavendulae REN-7:  
 Purification, Characterization, and Nucleotide Sequence

JOURNAL Unpublished

REFERENCE 2 (bases 1 to 2318)

AUTHORS Suzuki,T. and Ito,M.

TITLE Direct Submission

JOURNAL Submitted (26-SEP-2002) Takashi Suzuki, Rengo Co., Ltd., central  
 laboratory; 4-1-186, ohhiraki, fukushima-ku, osaka, osaka 553-0007,  
 Japan (E-mail:ta2-suzuki@rengo.co.jp, Tel:81-6-6466-7446,  
 Fax:81-6-6465-0220)

FEATURES Location/Qualifiers

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## NCBI Sequence Viewer

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Search  for

1: NM\_069101. Caenorhabditis el...[gi:17539949]

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LOCUS NM\_069101 2142 bp mRNA linear INV 15-SEP-2003  
 DEFINITION Caenorhabditis elegans laccase (4J499), mRNA.  
 ACCESSION NM\_069101  
 VERSION NM\_069101.1 GI:17539949  
 KEYWORDS  
 SOURCE Caenorhabditis elegans  
 ORGANISM Caenorhabditis elegans  
 Eukaryota; Metazoa; Nematoda; Chromadorea; Rhabditida;  
 Rhabditoidea; Rhabditidae; Peloderinae; Caenorhabditis.  
 COMMENT PROVISIONAL REFSEQ: This record has not yet been subject to final  
 NCBI review. The reference sequence was derived from WormBase  
 CDS:F21D5.3.  
 Summary: This gene 4J499, also known as F21D5.3 or YK674, maps at  
 (IV; +4.53). It encodes a laccase. From Pfam homology, the product  
 would have copper binding activity.

According to the Worm Transcriptome Project, it is expressed at  
 high level mostly from L2 larvae to adult [Kohara cDNAs]. Its  
 existence, but not its exact sequence, derived here from the genome  
 sequencing consortium annotation, is supported by 34 cDNA clones.

RNA interference results:  
 [A.Sugimoto 2000] No obvious phenotype (by injecting cDNA clone  
 SA:yk261a7).

## Expression

The expression profile for the gene, derived from the proportion of  
 animals at each stage in each Kohara library is: embryos 2%, L1 or  
 L2 larvae 12%, L3 to adult (including dauer) 86%.  
 In situ hybridisation pictures to all stages of development are  
 available from Kohara NextDB.

The predicted CDS has 14 exons. It covers 4.69 kb on the WS97  
 genome. The protein (713 aa, 81.7 kDa, pI 5.6) contains 3  
 multicopper oxidase, type 1 motifs. Taxblast (threshold 10<sup>-3</sup>)  
 tracks ancestors down to bacteria and eukaryota.

COMPLETENESS: full length.

## FEATURES

source

Location/Qualifiers

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## NCBI Sequence Viewer

Page

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